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Tapetal and parenchymatic anther tissues participate in polyad adhesive production in *Calliandra brevipes* (Leguminosae)

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ABSTRACT

Calliandra brevipes Benth. is an American shrubby species largely used for ornamental purposes. Like all other *Calliandra* species it has heteromorphic pollen grains shed in ellipse-shaped, calymmate polyads and a unique mode of pollen presentation by producing a sticky substance called "pollen adhesive". The present study aimed to investigate in detail the origin of polyad adhesive in *C. brevipes*. Serial microtome sections of anthers in various developmental stages were used and histochemical tests applied to detect the adhesive production sites and investigate the subcellular characteristics of the anther cells under transmission electron microscopy. The pollen adhesive in *C. brevipes* is not only produced by parenchymatic cells of the anther transversal septum, as it is described for *Calliandra angustifolia*, but parenchymatic cells and tapetal cells next to the polyad apical pollen grain also participate in the pollen adhesive production. The cytoplasm of the degenerating tapetum cells contains oleoplasts and fibrogranular material inside the vacuoles which mixes with the adhesive produced by adjacent parenchymatic cells and which therefore contributes to its composition. Vacuoles containing fibrogranular material are very similar to those found in tryphine producing tapetal cells, and the subcellular structures of parenchymatic and tapetal cells are similar to each other. The fact that the pollen adhesive becomes solid in contact with the environment is attributed to dehydration and the presence of the protein fibrogranular material and lipid substances since resin could not be found in its composition. It seems that the sites of pollen adhesive production in *Calliandra* may vary among its members. Studies of polyad adhesive production in the genus should be standardized in order to verify the information already available in the literature.

1. Introduction

Calliandra Benth. (Leguminosae, Mimosoideae, Ingeae) was revised by Barneby (1998), who excluded the African and Asian members in a way that, in its current circumscription, the group is exclusively Neotropical with about 130 species of trees and shrubs. Its representatives have flowers generally organized into paniculate racemes, with 10 to 100 stamens and their filaments are fused at the base, forming a tube at the non-exposed region (hidden by corolla) and free at the exposed region (Martius and Eichler, 1840–1906; Macqueen and Hernández, 1997). They have a unique mode of pollen presentation and produce a sticky substance called "pollen adhesive" (Prenner and Teppner, 2005; Teppner and Stabentheiner, 2007; Santos and Romão, 2008). Inside of each septate locule of the anther are two drop-shaped polyads which lie longitudinally and parallel to the anther axis (Prenner and Teppner, 2005). *Calliandra* polyads are calymmate and comprise eight heteromorphic pollen grains: two central grains surrounded by another six, one being the apical, which is different from the others in shape and position, forming an acute portion by which the polyad is attached to the anther by the pollen adhesive (Santos and Romão, 2008). These peculiar palynological characteristics distinguish *Calliandra* species from other genera with polyads in Mimosoideae (Greissl, 2006).

Pollen adhesive production in *Calliandra* seems to occur before the anther opening and is internal to the extra-tapetal layer (Prenner and Teppner, 2005; Teppner, 2007b). It has been considered a lysigenous product of the parenchymatic tissue within the locules (Prenner and Teppner, 2005), and stored in hemispherical cavities ("mucilage chambers") of the transversal septum formed by lysis of the anther middle layer cells (Teppner, 2007b). This information disproved the hypothesis that pollen adhesive could be produced by tapetum cells, such as the pollenkitt (Pacini and Hesse, 2005). Pollen adhesive is a lipophilic mixture composed of wax-like free fats and unsaturated fatty acids covered by a thin protein layer (Greissl, 2006). However, tests for resin substances produced by some Leguminosae species and used by many groups of bees to build their nests (Armbruster, 1984; Leonhardt and Bluthgen, 2009) were not performed.

The studies by Armbruster (1984), Mariani and Wolters-Arts (2000), Pacini and Hesse (2005), Leonhardt and Bluthgen (2009), and Hesse (2010) show that these sticky substances originating from plant reproductive structures, or from other structures associated with them, have many different functions related to pollination processes such as:

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enabling secondary pollen presentation; gluing pollen grains together at dispersion time; protecting pollen grains from excessive loss of water; increasing the attractiveness of pollen grains to animals or repelling the animals who eat them; and allowing pollen grain adhesion to the visitor body (pollinator).

The attractive flowers of *Calliandra* are visited by a wide range of pollinators, such as moths and butterflies (Haber and Fankie, 1989), hummingbirds (Arroyo, 1981) and bats (Chamberlain and Hubert, 2001). The structural characteristics of *Calliandra* polyads may have evolved in response to this variety of visitors, in particular the size of the polyad (considered big) and its sophisticated mode of transportation. Hence, studies regarding anther and polyad development and morphology in *Calliandra* species are becoming more frequent. Current literature provides ontogenetic studies for four species: *Calliandra angustifolia* Spruce ex Benth. (Prenner and Teppner, 2005), *Calliandra calothyrsus* Meisn, *Calliandra haematocephala* Hassk. and *Calliandra tweediei* Benth., and one synonym, *Zapoteca tetragona* (Willd.) H.M. Hern. (*Zapoteca* syn. *Calliandra* p.p.) (Greissl, 2006); pollen morphology studies for 21 species (herbarium samples): *Calliandra asplenioides* (Nees) Renvoize, *Calliandra bahiana* Renvoize, *Calliandra bella* (Mart. ex Spreng.) Benth., *Calliandra coccinea* Renvoize, *Calliandra depauperata* Benth., *Calliandra elegans* Renvoize, *Calliandra ganevii* Barneby, *Calliandra germana* Barneby, *Calliandra harrisii* (Lindl.) Benth., *Calliandra hirsuticaulis* Harms, *Calliandra hirtiflora* Benth., *Calliandra hygrophila* Mackinder & G.P. Lewis, *Calliandra lanata* Benth., *Calliandra leptopoda* Benth., *Calliandra lintea* Barneby, *Calliandra macrocalyx* Harms, *Calliandra semisepulta* Barneby, *Calliandra sessilis* Benth., *Calliandra spinosa* Ducke, *Calliandra stelligera* Barneby and *Calliandra viscidula* Benth. (Santos and Romão, 2008); and studies of floral morphology, anther opening patterns and pollen presentation for four species: *C. angustifolia*, *C. haematocephala*, *Calliandra tergemina* (L.) Benth. and *C. tweediei* (Teppner, 2007a, b; Teppner and Stabentheiner, 2007).

With the goal of expanding our knowledge of the pollen adhesive origin, we studied the ontogeny of polyads and anthers in *Calliandra brevipes* Benth., an ornamental species, widely distributed in Brazil (Lorenzi and Souza, 1995), emphasizing subcellular features of the cells adjacent to the anther locule.

2. Material and methods

Buds of various sizes and flowers of *C. brevipes* were collected, fixed in Karnovsky in phosphate buffer 0.1 M (pH 7.3) for 24 h (Karnovsky, 1965) and in FAA 50 for 24 h (Johansen, 1940), dehydrated in ethanolic series and stored in 70% alcohol.

Anatomic studies of the developing anther wall and pollen grains were made with anthers from buds in various development stages, previously fixed in Karnovsky in phosphate buffer 0.1 M (pH 7.3) for 24 h (Karnovsky, 1965), embedded in historesin (Gerrits, 1991) and

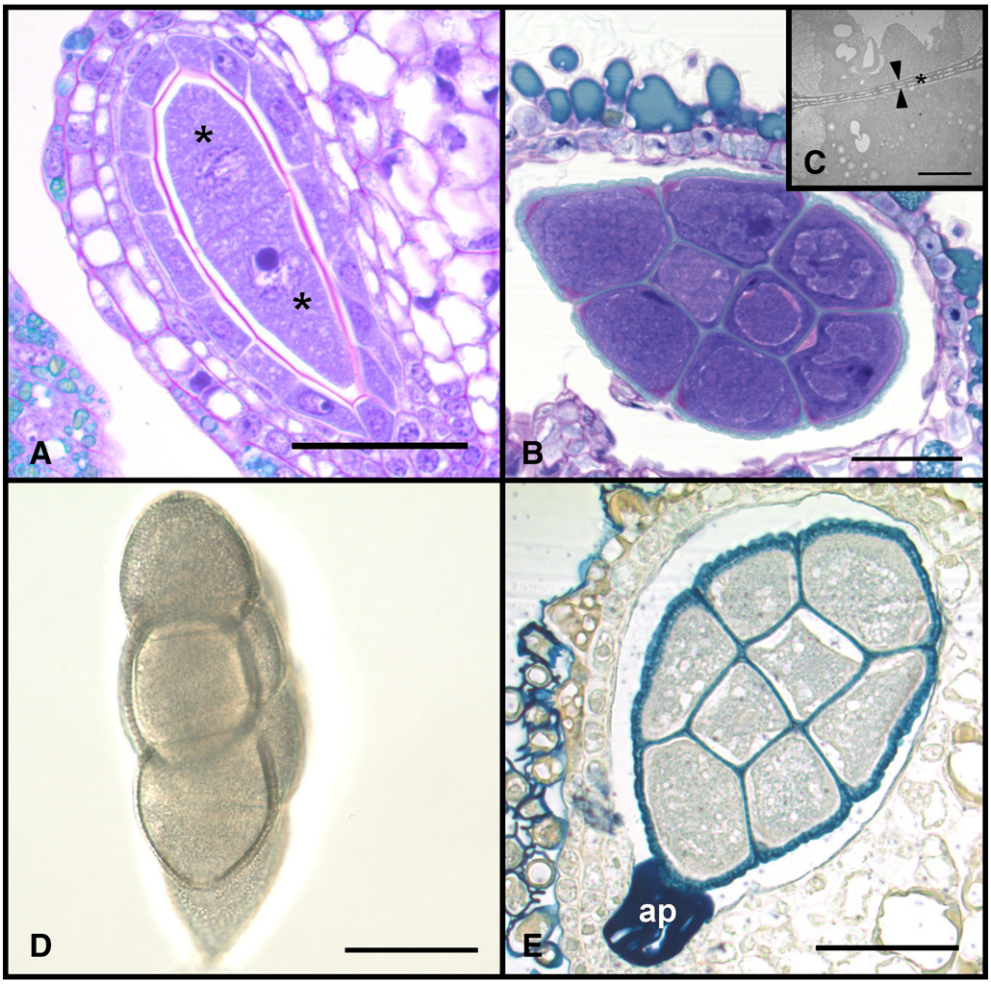

Fig. 1. *Calliandra brevipes* pollen mother cells and polyads under light microscopy (A and B, Toluidine blue staining; D, fresh and untreated material in immersion oil; and E, Sudan black B staining) and transmission electron microscopy (TEM, C). A. Pollen mother cells (*). B. Ellipsoidal, drop-shaped polyad formed by eight pollen grains. C. Contact region between pollen grains in the inner portion of the polyad, showing the exine (calymmate polyad). Note presence of endexine (arrow heads), columellae and tectum (*). D. Polyad lateral view showing its flattened shape, with only one layer of pollen grains. E. Viscous pollen adhesive (ap) attached to the apical pollen grain of the polyad in pre-anthesis anther. Scale bars: A, B, D, E = 50 μm; C = 10 μm.

sectioned transversally and longitudinally using a Leica RM2245 microtome at 3 μm. Sections were stained with Toluidine blue 0.01% pH 4.4 (O'Brien et al., 1964), observed and illustrated under a Leica DM5000B light microscope.

Histochemical tests were performed to locate the production sites of the pollen adhesive substances in anatomical sections, using PAS reagent to detect carbohydrates (Feder and O'Brien, 1968), xylidine de Ponceau to detect proteins (O'Brien and McCully, 1981) and Sudan black B for lipids (Pearse, 1980). Presence of resin in pollen adhesive composition was tested, using fresh samples of anthers gently macerated in Cupric acetate at 7% (Johansen, 1940) with a glass rod to expose the polyads from the inside of anthers. The samples were left for 15 to 25 min in the solution and observed under light microscopy. Positive control was made with *Pinus* sp. leaves containing resin.

Ultrastructural characteristics of the anther wall cells and polyad pollen grains were verified by means of transmission electron microscopy

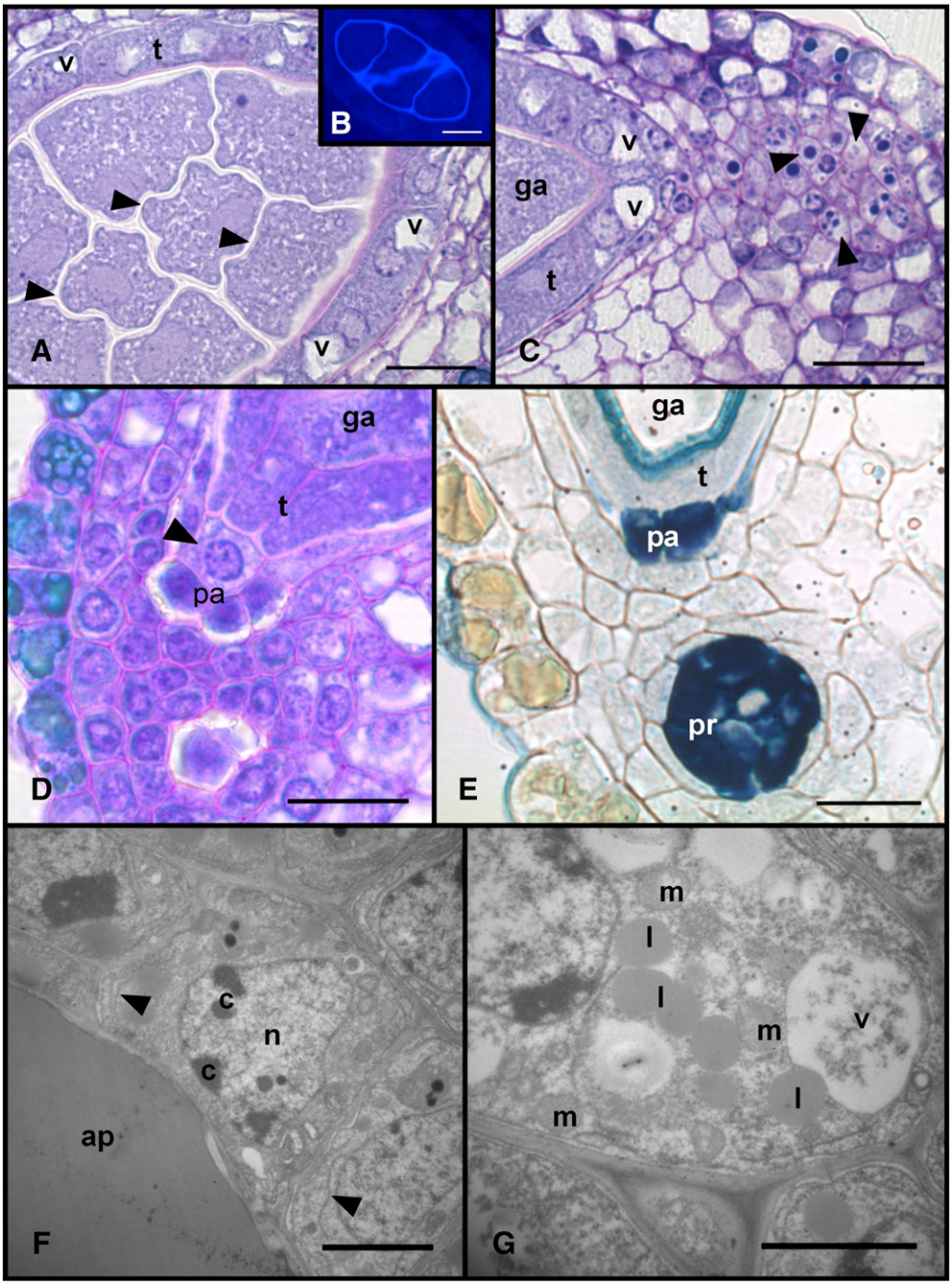

Fig. 2. Polyads and anther cells of *Calliandra brevipes* under light microscopy (A, C and D, Toluidine blue staining; B, Aniline blue staining — fluorescence; E, Sudan black B staining) and transmission electron microscopy (TEM, F and G). A. Androspores surrounded by callose (arrow heads). Tapetum cells (t) are starting to degenerate (presence of vacuoles — v). B. Fluorescing callose wall. C. Transversal septum region of the anther showing cells with high metabolic activity, represented by its conspicuous nucleus and manifold mitotic figures (arrow heads). Note the proximity of these cells in relation to the locule, at the apical pollen grain region (ga) surrounded by tapetal cells (t) containing vacuoles (v). D. Cells of the adjacent parenchyma (pa) showing conspicuous nucleus and densified chromatin (arrow head). Tapetum (t) is still cellularized next to the polyad apical pollen grain (ga). E. Adjacent parenchyma (pa) immediately next to tapetum (t) and to the apical pollen grain (ga) of the polyad, presenting initial pollen adhesive accumulation, composed mainly of lipids (stained in blue). The same phenomenon is observed in anther transversal septum parenchymatic cells (pr). F. Pollen adhesive-producing cell showing conspicuous nucleus (n) with densified chromatin (c) and many smooth and rough endoplasmic reticulum in its cytoplasm (arrow heads). Note the pollen adhesive accumulation (ap). G. Pollen adhesive-producing cell with large number of mitochondria (m) and droplets of lipids (l) in its cytoplasm. Note the large vacuole containing fibrogranular material (v). Scale bars: A, B, C, D, E = 20 μm; F, G = 3 μm.

(TEM). To do so, part of the Karnovsky fixed material (Karnovsky, 1965) was post-fixed in Osmium tetroxide at 1% (phosphate buffer 0.1 M and pH 7.3) for 2 h, dehydrated in a graded acetone series and embedded in Araldite 502 Polysciences epoxy resin. Semi-thin sections (0.5 μm) were obtained using a Leica ultracut S Reichert ultramicrotome, stained with 0.05% Toluidine blue (O'Brien et al., 1964) and observed under light microscopy. Ultra-thin sections (60 to 70 nm) were collected on a fine mesh grid, contrasted in 2% uranyl acetate in aqueous solution for 15 min (Watson, 1958) and lead citrate for 15 min (Reynolds, 1963). Analyses and illustrations were made using a Philips EM 208 transmission electron microscope.

Terminology in this study follows Prenner and Teppner (2005) and Hesse et al. (2009).

3. Results

Polyads of *C. brevipes* originate from two pollen mother cells (Fig. 1A) that give rise to one tetrad each, resulting in one drop-shaped polyad with eight pollen grains (Fig. 1B). Polyads are calymmate (Fig. 1C) and flattened (Fig. 1D) and one of the peripheral pollen grains (apical grain) forms an acute apex, where slightly before anthesis and anther opening a sticky substance is deposited (Fig. 1E).

After complete formation of the androspores, when each one is still surrounded by callose wall and tapetum cells are still intact although showing vacuoles — the beginning of the degeneration process (Fig. 2A–B), transversal septum cells of the anther present high metabolic activity, showing conspicuous nucleus, or many of them, in addition to mitotic figures (Fig. 2C).

Then, when the pollen wall deposition is complete and callose walls are dissolved, parenchymatic cells close to the locule and immediately next to the tapetum cells also present high metabolic activity, showing conspicuous nucleus and chromatin densification inside (Fig. 2D). At this stage, both transversal septum cells and cells adjacent to the locule start the production and accumulation of pollen adhesive (Fig. 2E).

Pollen adhesive-producing cells present conspicuous nuclei with densified chromatin, a cytoplasm which is rich in rough and smooth endoplasmic reticulum (Fig. 2F) and which contains mitochondria, lipids and vacuoles with fibrogranular material (Fig. 2G).

Pollen adhesive accumulation takes place at the intercellular spaces of the parenchyma (Fig. 3A). As the accumulation increases (Fig. 3B), producing cells initiate vacuolation process (Fig. 3C) and the vacuole rapidly becomes larger, dislocating the cytoplasm to the periphery, close to the cell wall (Fig. 3D).

At the end of the vacuolation process, producing cells undergo lysis, forming a linking-space between adhesive-producing parenchymatic

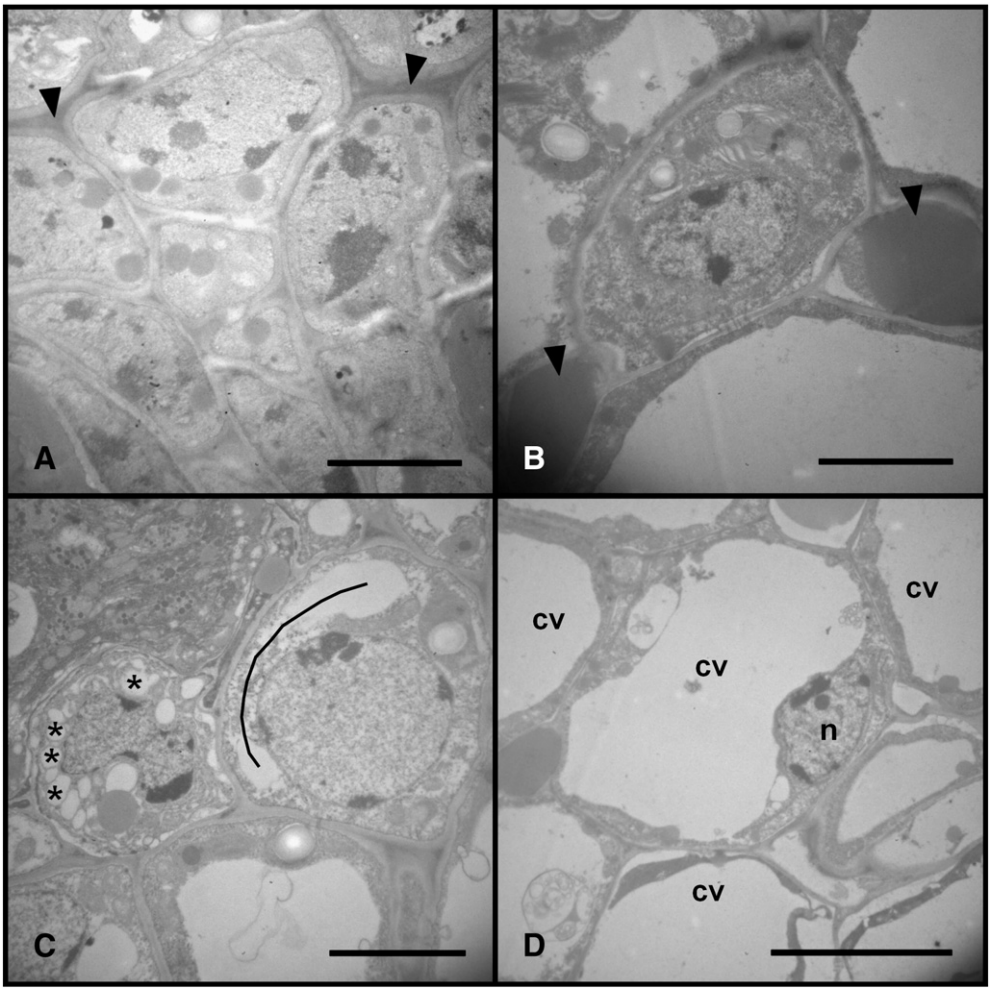

Fig. 3. Transversal septum with parenchymatic cells of the anther, and pollen adhesive producing cells in *Calliandra brevipes* (TEM). A. Early accumulation of pollen adhesive in the intercellular spaces (arrow heads). B. Advanced stage of adhesive accumulation. Large amount of pollen adhesive has accumulated in the intercellular spaces (arrow heads). C. Beginning of the vacuolation process in adhesive producing cells. Note the presence of small vacuoles (*) in the cytoplasm that later coalesce, forming only one large vacuole (line). D. Adhesive producing cells in advanced stage of vacuolation (cv). Note the cell nucleus leaning to the cell wall (n). Scale bars: A, B, C = 5 μm; D = 10 μm.

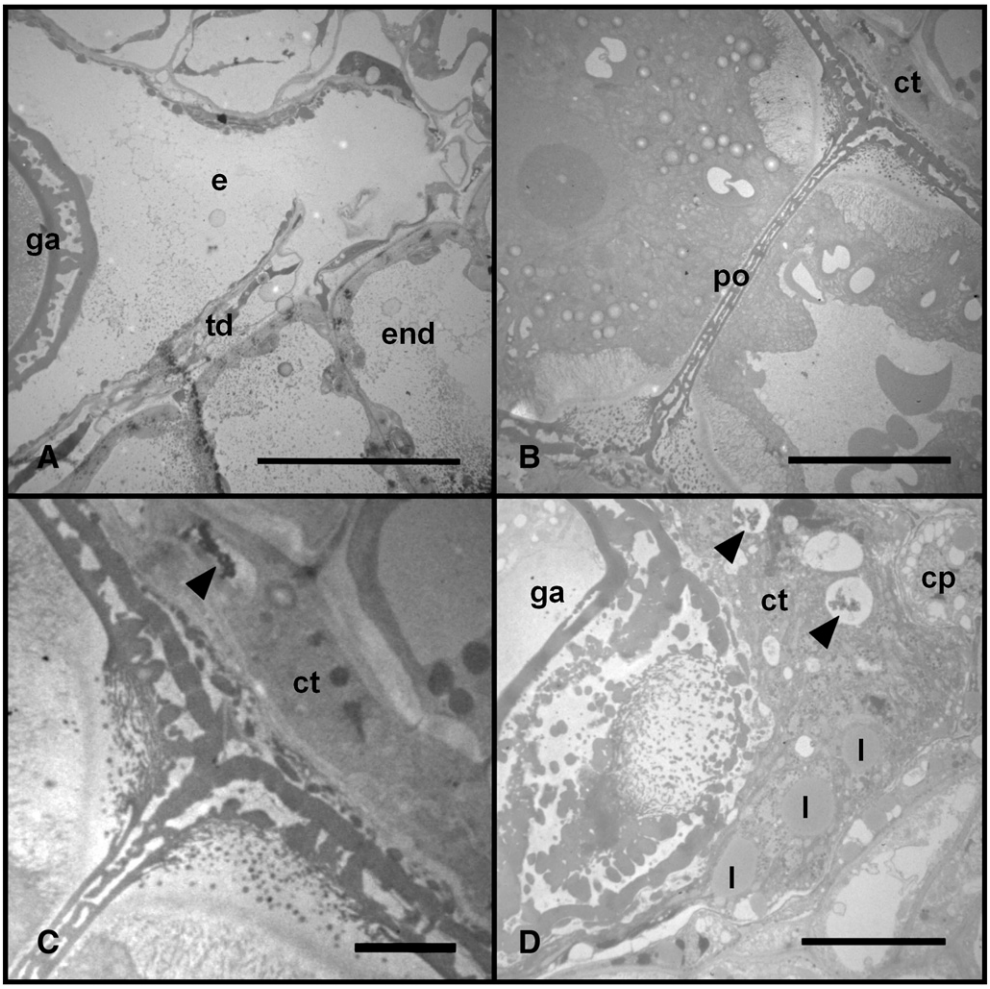

Fig. 4. Anther locule region of *Calliandra brevipes* (TEM). A. Space (e) resulting from degeneration of the parenchymatic cells adjacent to the locule and transversal septum cells of the anther, linking the adhesive accumulation region to the apical pollen grain (ga). Note remnants of the middle layers (td) near to the endothecium (end). B. Cytoplasmic content (ct) of the degenerating tapetum on the side of the polyad (po). C. Degenerating tapetum cytoplasmic content (ct) showing vacuoles with fibrogranular material inside (arrow head). D. Locule region next to the apical pollen grain (ga) filled with cytoplasmic content (ct) of the tapetum showing droplets of lipid (l) and vacuoles containing fibrogranular material (arrow heads). Note adhesive-producing cells (cp) in which the degeneration process (lysis) starts by vacuolation. Scale bars: A, B = 20 μm; C = 5 μm; D = 10 μm.

region and the locule, allowing pollen adhesive to overrun it (Fig. 4A). Thereby, cytoplasmic content of the degenerating tapetum cells (non-cellularized) (Fig. 4B), containing fibrogranular material inside vacuoles (Fig. 4C, D — see arrow heads) and droplets of lipid (Fig. 4D — labeled as l) and, very similar to those found in parenchymatic producing cells, mixes with the pollen adhesive and contributes to its composition.

Chemical tests indicated that the pollen adhesive comprises mainly lipids and small amounts of carbohydrates (Fig. 5A–B). Proteins and resin substances were not detected (Fig. 5C–D).

4. Discussion

We found that, unlike *C. angustifolia* (Prenner and Teppner, 2005; Teppner, 2007b), pollen adhesive of *C. brevipes* is not only produced by parenchymatic cells of the anther transversal septum but also by parenchymatic cells adjacent to the locule at the apical pollen grain region. Moreover, the tapetum also contributes to the chemical composition of the pollen adhesive since its cytoplasm mixes with the adhesive substance produced by the adjacent parenchymatic cells. Therefore, considering that many similarities were found, pollen adhesive in *C. brevipes* can be compared with other two adhesive substances, the pollenkitt and the tryphine (Dickinson and Lewis, 1973; Pacini and Hesse, 2005), which are the main pollen coat forms known so far (Hesse, 2010).

Pollenkitt and tryphine are similar to each other in many respects because both substances are formed by fusion of elaiosomes and spherosomes produced in tapetum cells (Platt et al., 1998). Unlike these substances, no plastids participate in the formation of the lipid droplets of the elastoviscin, an adhesive substance present in Orchidaceae species also produced by tapetum cells (Pacini and Hesse, 2005; Hesse, 2010). Regarding the pollen adhesive in *Calliandra*, it is more similar to the tryphine than to the pollenkitt because organelles, cytoplasmic remnants and lipids from degenerated tapetum cells participate in its composition, while pollenkitt has only the lipidic fraction of the tapetum cell plastids and endoplasmic reticulum in its composition (Hesse, 2010). Furthermore, the cellular machinery of the pollen adhesive-producing cells in *C. brevipes* is quite similar to the subcellular features of the tryphine-producing tapetum in *Raphanus* (Brassicaceae) (Dickinson and Lewis, 1973).

Tryphine is composed of substances released inside the anther locule when tapetum cells disintegrate. It fills the cavities of the pollen wall between the columellae (bacula) of the exine (Mariani and Wolters-Arts, 2000), and covers the pollen grains in the same way as the pollenkitt (Pacini and Hesse, 2005). Its function in relation to the pollination process and pollen–stigma recognition has been discussed by Dickinson and Lewis (1973) and Mariani and Wolters-Arts (2000). In *Arabidopsis*

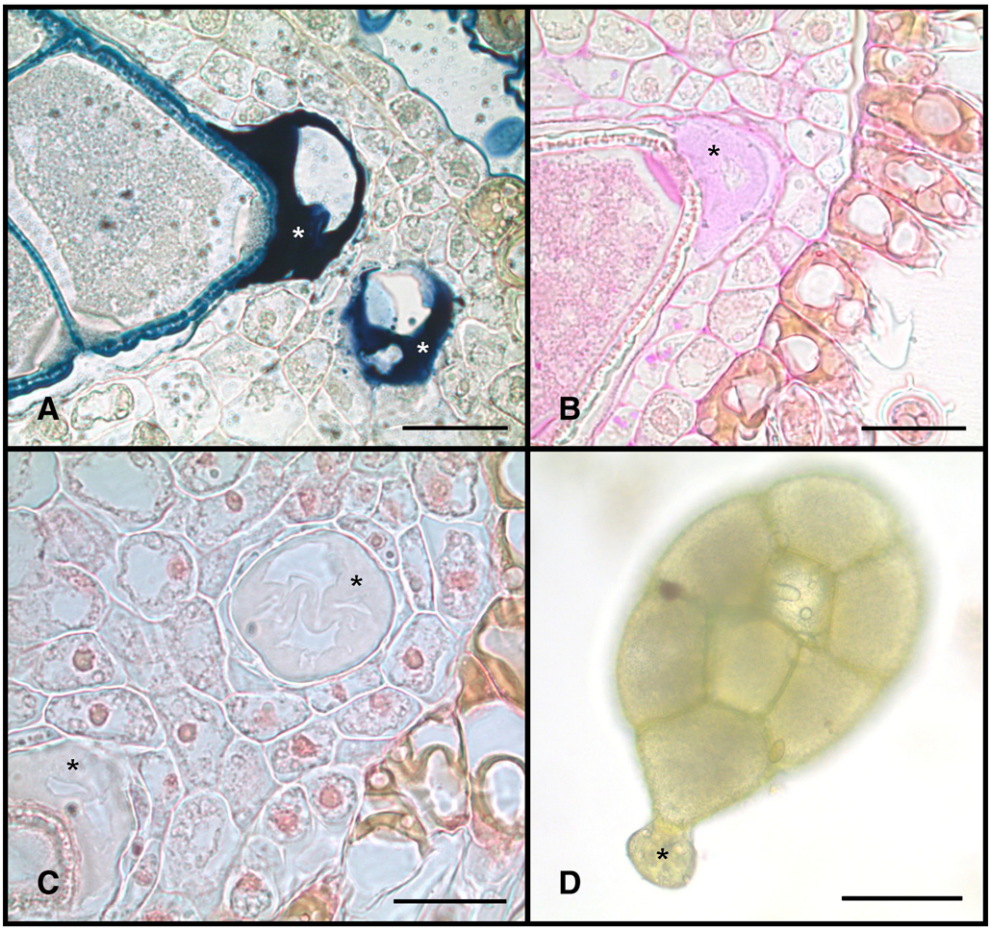

Fig. 5. Pollen adhesive in polyads of *Calliandra brevipes* (A, Sudan black B staining; B, PAS reaction; C, xylidine de Ponceau staining; D, fresh living material in cupric acetate at 7%). A. Pollen adhesive showing positive reaction for lipids (*). B. Pollen adhesive showing low positive reaction for carbohydrates (*). C. Pollen adhesive showing no reaction for proteins (*). D. Pollen adhesive showing no reaction for resin substances (*). Scale bars: A, B, C = 20 μm; D = 50 μm.

and other Brassicaceae, a tryphine conversion forms a lipid and protein foot between the pollen grain and the papillae of the stigmatic surface and establishes a hydraulic contact that allows pollen rehydration and germination (Mariani and Wolters-Arts, 2000). However, in *C. brevipes* the adhesive deposition occurs only on the apical pollen grain, suggesting that its ecological function is only related to polyad transportation (adherence to the pollinator body) and secondary pollen presentation. The polyad adherence to the anther during the first polyad presentation (early anthesis) seems to be the responsibility of the pollenkitt found on the polyad surface (Teppner, 2007b). The polyad of *Calliandra* is considered large, which could impede its transportation success if it was not aerodynamically shaped (flattened) and if it did not have the pollen adhesive, which guarantees the transfer of the pollen (usually shed in small amounts in this genus) to the stigmas (Greissl, 2006).

No reports were found in the literature of pollen adhesive which is comprised of derivatives from tapetum cells mixed with derivatives from extra-tapetal cells. Nevertheless, a rare type of adhesive substance, different from pollenkitt and tryphine, is found in some Araceae (monocot) species, originating not only from tapetum cells but also from locular substances during pollen mother cell stage, forming the so called "pollen droplets" or "pollen-strands" (Hesse, 2010).

Although protein was not detected in pollen adhesive of *C. brevipes* in histochemical tests, a fibrogranular material very similar to those found in tryphine composition with proteinaceous nature was revealed by ultrastructural analysis (Dickinson and Lewis, 1973). Furthermore, ontogenetic studies made by Greissl (2006) demonstrated that pollen adhesive, wrongly called "viscin body" in his study, contains small amounts of protein forming a thin layer covering the wax-like mixture (Greissl, 2006). The presence of this substance seems to maintain adhesive structure after its dehydration and stiffening provided by its contact with the air during anthesis, when *C. brevipes* polyads are exposed, such as in tryphine (Dickinson and Lewis, 1973), since resin substances could not be detected in the performed tests.

Our data show that the sites of pollen adhesive production in *Calliandra* may vary among species, perhaps depending on the pollinator behavior and the way the flower is exposed in the plant. However, we believe that studies of polyad adhesive production in the genus should be standardized in order to verify the information already available in literature.

Acknowledgments

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